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ORIGINAL CONTRIBUTION

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Erythritol ingestion impairs adult reproduction and causes larval mortality in *Drosophila melanogaster* fruit flies (Diptera: Drosophilidae)

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Abstract

Previous feeding studies showed the polyalcohol erythritol was toxic when ingested by adult laboratory fruit flies (*Drosophila melanogaster*). We asked whether erythritol could additionally affect fly population growth either through larval toxicity or through effects on adult reproduction. Females did not avoid laying on food substrates with 1M erythritol; laying rate on 1M erythritol food was similar to control food when females were given free-choice access. Eggs laid or placed on 0.5 M to 2.5 M erythritol foods hatched at normal rates, suggesting erythritol was not toxic to eggs upon contact. *Drosophila melanogaster* larvae readily consumed food containing 1 M erythritol, but none of these larvae reached pupation. Longevity of larvae feeding on in 1 M erythritol food was significantly reduced relative to controls, and mean ± *SE* larval lifespan on erythritol was 1.54 ± 0.10 days (max. = 3 days). Exposing cohorts of second-instar larvae to food with varying concentrations of erythritol showed the LD50 (at 24 hr) concentration was approximately 0.6 M. Taken together, these results suggest erythritol could be employed in effective larval-sink baits. Adults flies fed with erythritol produced significantly fewer eggs on days when they fed on 1 M erythritol, and egg production was significantly reduced for one additional day after the adults were moved to control food. These findings suggest erythritol is rapid and effective at temporarily suppressing *D. melanogaster* reproduction, increasing its potential for use in effective insect population control.

KEYWORDS

human-safe insecticide, larval sink, polyol, reproductive suppressor

1 | INTRODUCTION

Recent studies showed the polyalcohol meso-erythritol (henceforth erythritol) caused increased mortality when consumed by adult *Drosophila melanogaster* laboratory fruit flies (Baudier et al., 2014; O'Donnell, Baudier, & Marenda, 2016) and *Bactrocera* flies (Zheng, Zeng, & Xu, 2015). These findings suggest erythritol has potential for use as a human- and mammal-safe insecticide. Erythritol has low or no toxicity to humans and several other mammal species (Bernt, Borzelleca, Flamm, & Munro, 1996; Dean, Jackson, & Greenough, 1996; Noda & Oku, 1992; Storey, Lee, Bornet, & Brouns, 2007) and is approved for human food consumption (Cock, 1999). Erythritol is widely used as a commercial non-nutritive sweetener.

Compounds that suppress insect reproduction and compounds that control immature holometabolous insects can complement adult-stage insecticides and contribute to insect population control efforts (Barik, 2015; Campion, 1972). Sampson, Werle, Stringer, and Adamczyk (2016) showed erythritol added to *Drosophila* foods decreased pupation rates in culture vials in a dose-dependent manner. In this study, we tested whether erythritol could affect population growth either via reproductive suppression (egg laying) or larval mortality. First, we asked whether flies would oviposit on food containing

erythritol and whether egg contact with erytritol affected hatching. We then asked whether larval survival was affected by feeding on erythritol. Finally, we fed erythritol to adults and asked whether egg production and development of eggs from exposed parents were affected. We show that erythritol: (i) was ingested by larvae, (ii) prevented larval development (caused larval mortality) when ingested by larvae, and (iii) temporarily disrupted egg production by adults. These findings suggest erythritol could have pest control applications via insect population suppression that extend beyond adult toxicity/mortality.

2 | MATERIALS AND METHODS

2.1 | Culturing *Drosophila*

We used standard *Drosophila* food for laboratory culturing to rear flies to adulthood and as base food for the experimental treatments (Chakraborty et al., 2011). To obtain research subjects, we reared wild-type (Canton S) *D. melanogaster* larvae on food prepared in 100-ml batches as follows: 12-g cornmeal (LabScientific, Livingston, NJ, USA: FLY-8009-10), 4.8-g yeast (LabScientific 8030-5), 0.9-g agar, 12 ml molasses (LabScientific FLY-8008-4), 2.4-ml Tegosept (10% w/v methyl p-hydroxybenzoate in 95% ethanol) and 0.95 ml propionic acid with 84 ml of distilled water. Cornmeal and yeast in the base food assured flies received sufficient carbohydrates and protein in addition to any effects of the treatment additives. After heating each batch of food to set the agar, foods were poured into vials and cooled until consistency was firm. Each treatment rearing vial contained 10 ml of food, such that food was provided in excess in all cases. Food consistency was uniform across treatments: foods did not run when vials were placed on their sides. All flies were reared in an insect growth chamber set at 25°C, under a 12-h:12-h photoperiod, and at 50% relative humidity.

2.2 | Testing erythritol for egg-substrate contact effects and effects on larval development

We placed groups of 50 mixed male and female flies in each of 10 egg laying chambers to produce the eggs used as subjects. Each laying chamber was a single-standard fly rearing vial inverted atop laying substrate in a 35 × 10-mm Petri dish. Each Petri dish was loaded with an extra-firm food substrate to induce egg-laying and allow egg collection. Laying substrate comprised molasses, distilled water, propionic acid and agar, with approximately 0.5 ml of semi-solid yeast whip atop the food surface in the centre of the dish to provide additional protein for the adult flies during laying (modified from Featherstone, Chen, & Broadie, 2009). Yeast whip was produced by mixing dry yeast with water at approximately even weight:volume to produce a moist but firm mass. After four hours elapsed, $N = 50$ eggs 0- to 4-h old were collected from the laying chambers and transferred to each of $N = 6$ rearing vials/treatment, for a total of $N = 300$ eggs/treatment. Treatment foods in the rearing vials consisted of either of two treatments: control fly food or food with 1 M erythritol added. We surveyed each rearing vial every 24 hr. During every survey, we counted the number of hatched eggs, number of pupae and number of adult files that had emerged. We continued these surveys for 14 days from introducing the eggs.

We followed up with a test of erythritol concentration effects on egg hatching. We placed 10 eggs on each of three replicate plates/treatment ($N = 30$ eggs/treatment) with treatments including control food and food with erythritol concentrations every 0.5 M from 0.5 M to 2.5 M. We examined the eggs for successful hatching after 24 hr.

2.3 | Testing for oviposition avoidance by females

Mixed groups of adult male and female flies 0–24 hr from adult eclosion were placed on control food for 72 hr before introduction to the egg-laying choice experiment. This provided adequate time for mating and allowed us to sample egg laying preferences at the age of peak of fertility. The arena used for egg laying choice was similar to that used for testing adult food choice by Baudier et al. (2014) and consisted of two standard fly rearing vials placed on their sides. Vials were connected by a 1.5-cm perforated plug, allowing flies to pass from one side to another. Previous testing with blue-dyed food showed that flies moved freely between the vials in this set-up (Baudier et al., 2014). On day 3 of adulthood, 10 male and 10 female flies were transferred to choice treatment paired vial set-ups with 1M erythritol in one vial and control food in the other vial. Within each treatment, the flies were moved to a new food arena every 24 hr. Ten flies (5 males, 5 females) were placed on either side of the plug every time flies were moved to fresh food arenas. Eggs laid on each food were counted daily. The arenas were kept in an incubator set to 25°C, 12:12 light dark and 50% relative humidity. Tube orientations within the incubator were rotated daily. We conducted four trials. Flies were allowed to lay eggs for 4 days in trial one and 3 days in the other trials.

2.4 | Testing for larval food avoidance with brilliant blue food dye

We obtained subject eggs as in Study 1. Eggs were placed 0–4 hr after laying on food without molasses and with 0.05% Brilliant Blue FCF dye added (Fisher Scientific Cat# BP101) in two separate treatments: food with 1 M sucrose (control) versus food with 1 M erythritol. Six rearing vials were used per treatment, with 50 eggs per vial, for a total of $N = 300$ eggs per treatment. The food surfaces in each vial were surveyed for hatched eggs, and visible larvae at 24 hr and again at 48 hr after the eggs were introduced. Not all larvae were visible during the surveys as many were burrowing within the food, but the number of larvae visible and active on the surface was recorded. We noted whether larvae were moving and apparently feeding, and we also scored whether blue food was visible in each larva's gut through the translucent body wall. Blue material (dyed food) visible in the larval gut was taken as evidence the larva was ingesting the food.

2.5 | Determining adult LC50 at 24 hr

Subject *Drosophila melanogaster* (Canton S) were reared on standard food. Treatment foods contained 0.05% Brilliant Blue R-250 food dye (to visually check flies for food ingestion); we used control food and foods with erythritol in 0.5 M concentration increments from 1 M to 2.5 M. Foods were poured into standard fly rearing vials with three replicates per treatment. Subjects were 150 flies that were collected 24–48 hr after adult eclosion; 10 flies (five males, five females) were placed in each of the culture tubes. Flies fed freely for 24 hr. At the end of 24 hr, dead flies were counted. Mean % mortality per concentration and standard error were calculated from three replicates each (N = 10 flies per vial). A three-parameter sigmoid curve was fitted to the mean adult mortality (24 hr) vs. erythritol concentration data to estimate the adult LC50 via interpolation.

2.6 | Determining larval LC50 at 24 hr

Translucent food was produced by omitting the cornmeal from the standard food recipe above. Food was poured to a depth of approximately 3-mm into 50-mm-diameter plastic Petri dishes. Treatments included control food and foods with erythritol in 0.5 M concentration increments from 0.5 M to 2 M. We obtained larvae from Canton S fly eggs laid on caps with yeast whip (as above). After hatching, larvae were reared 72 hr until the mid-second-instar stage on control food without erythritol. The second-instar larvae (N = 3) were placed on each of three replicate food plates per treatment concentration (N = 9 larvae/treatment). Larval mortality was recorded 24 hr after introduction to treatment foods. A three-parameter sigmoid curve was fitted to the mean larval mortality (24 hr) vs. erythritol concentration data to estimate larval LC50 via interpolation.

2.7 | Determining larval longevity feeding on 1M erythritol

Approximately 400 mixed male and female adult CantonS were placed on egg lay caps with yeast whip (as previously described); 100 eggs were collected 0–2 hr after having been laid, and placed on control and 1M erythritol foods clear foods in 50-mm Petri dishes as described above. Sample sizes were five eggs per plate and 10 plates per treatment. Number of hatched eggs was counted after 24 hr, and number of live larvae was counted every 24 hr from day 1 until pupation. To survey larval mortality, we examined the plates under a dissecting scope with a fibre-optic illuminator at 20 X-40 X. Larvae that were immobile were gently prodded; if they did not respond, they were counted as dead. No larvae scored as dead moved subsequently or developed further. To verify the efficacy of these foods for supporting normal development, the control group flies were allowed to proceed to pupation.

2.8 | Timing of adult ingestion of erythritol and effects on reproduction

Parental flies raised on stock food until the pupal stage were used as subjects. We collected virgin adult flies (0–12 hr post-eclosion) and placed them in rearing vials one of two types of foods: stock food, or stock food with 1 M erythritol. Each treatment had three replicate vials with N = 5 males and N = 5 females originally placed in each vial (N = 30 adult flies/treatment; total N = 120 adults). All surviving adult flies in each vial were moved together to a new rearing vial every 24 hr throughout the egg collection part of the experiment, which lasted 7 days. The schedule of presenting the food types to the adults followed four treatments: control (stock food only) for 7 days, erythritol food on day 1 only and stock food on all other days, erythritol food on day 2 only and stock food on all other days, and erythritol food on days 2-7 (stock food on day 1 only). Adult survival and number of eggs laid were monitored every 24 hr. On each day the cohort of eggs were left in place in their home vial after the adults had been moved to the new vial. Each vial's eggs were monitored daily for an additional 14 days after laying; two weeks is beyond the expected time to adult emergence under our rearing conditions (Zwaan, Bijlsma, & Hoekstra, 1995). Within each vial, we recorded the number of eggs hatching, the number of pupae and the number of adults emerged every 24 hr.

2.9 | Statistical analyses

Analyses were performed using SPSS v. 24 software (IBM corp. 2016) and Sigmaplot v. 12.5 software. For the egg-contact and larval survival studies, we used survival analysis to test for treatment effects on the timing (in days) of egg hatching and pupation in the egg-contact study, and age of death in the longevity study. Eggs that did not hatch or pupate by the end of observations, or flies still alive at the end of the observations, were included in the analyses as right-censored values assigned to the last day of observations. Treatment differences in timing distributions were tested using the log-rank (Mantel-Cox) test.

The egg laying substrate choice data were analysed using nested ANOVA, with treatment (erythritol vs. control) nested within trial. The files were tested at their peak reproductive output ages, and egg laying rates did not show obvious directional trends over time, so we analysed the number of eggs laid on each day within a trial as independent data points.

Data on egg hatching and larval feeding from the blue-dyed food study were analysed using independent-samples t tests to compare the mean number of eggs hatched and mean number of larvae visible on days 1 and 2 of observations; rearing vials were treated as replicates (N = 6 per treatment). We analysed two response variables to test whether adult exposure to erythritol affected egg production: the total number of eggs produced by the end of egg collections (day 7) by each group of flies (this measure assesses treatment effects on both reproduction and adult fly mortality), and the total number of eggs produced per living female in each group of flies (this measure assesses only effects on reproduction and is independent of adult mortality). We analysed the effects of treatment (control, and erythritol on day 1 only, day 2 only, and days 2-7) using generalized linear Models (GLM), with Tukey post hoc tests (critical alpha = 0.05) used to compare treatment means. Rearing vials (N = 3 per treatment) were treated as replicates in the analysis.

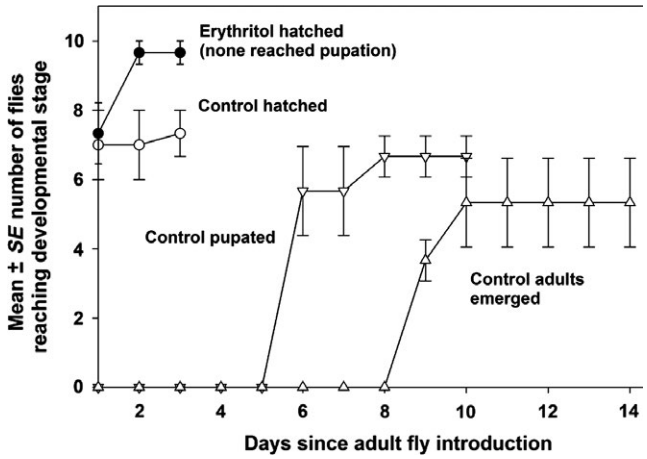

FIGURE 1 Time course of developmental events for fruit flies exposed to control and 1M erythritol feeding treatments. Note that no larvae reached pupation in the erythritol food treatment

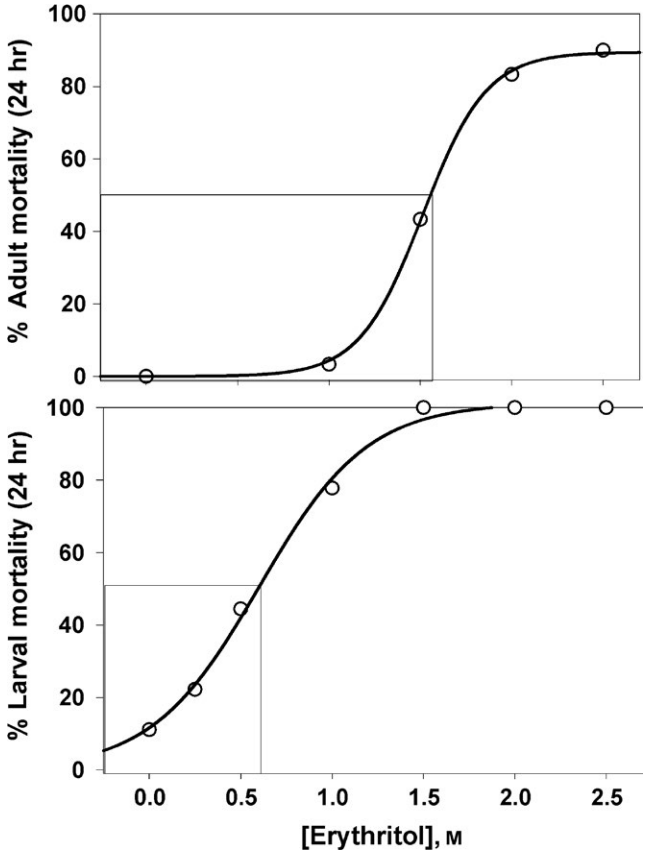

FIGURE 2 Per cent survival at 24 hr plotted against concentration of erythritol in foods provided to fruit flies. The three-parameter best-fit sigmoidal function is shown in each plot. Vertical and horizontal plotted lines indicate the [erythritol] corresponding to 50% survival in each case. Top: LC50 at 24-h data for adult flies; bottom: LC50 at 24-h data for larval flies

We estimated the LC50 (at 24 hr) of adult and larval flies by plotting Pr(flies alive) at 24-h versus erythritol treatment concentration. We used the three-parameter sigmoidal curve fitting function in Sigmaplot v. 12.5 to calculate the best-fitting 24-h survival/concentration curves for larvae and adults separately, then we interpolated to estimate the erythritol concentration that would yield 50% survival at 24 hr (LC50).

To test whether the eggs laid by females that had ingested erythritol exhibited developmental effects, we used GLM to test whether the proportion of individuals reaching developmental milestones differed between treatments, with three replicates (rearing vials) in each treatment. For each rearing vial, we calculated eggs hatched/eggs laid, pupae produced/eggs hatched and adults emerged/pupa produced. We tested for treatment effects on each developmental step separately.

3 | RESULTS

3.1 | Erythritol egg-contact effects on hatching and larval development

Contact with food substrate containing 1 M erythritol did not inhibit egg hatching. Eggs placed on food substrate with 1 M erythritol hatched at marginally significantly higher rates than eggs placed on control food (Figure 1; survival analysis Mantel-Cox test, $X^2 = 3.94$, $df = 1$, $P = .047$). However, no erythritol-contact larvae pupated by the end of observations at 14 days, a highly significant effect (Figure 1; survival analysis Mantel-Cox test, $X^2 = 36.99$, $df = 1$, $P < .001$). Tests of varying erythritol concentrations (0–2.5 M erythritol in 0.5 M increments) on hatching rates showed a non-significant trend towards decreased hatching success at higher erythritol concentrations ($r = -.46$, $N = 18$, $P = .058$).

In the comparison of larval survival on thin layers of clear food with control and 1M erythritol treatments, mean ± *SE* hatching rates on control food (78 ± 4.7%) and 1M erythritol (74 ± 6.7%) were similar (*T* test, $t = 0.49$, $df = 18$, $P = .63$). The larvae on control food had highly significantly greater survival in days from day of hatching (Log-rank test, $X^2 = 63.1$, $P < .001$). Mean ± *SE* longevity of larvae on 1M erythritol was 1.54 ± 0.10; only one subject out of 39 live to day 3 and none reached pupation. In contrast, 90% of control larvae had pupated at the end of observations on day eight, showing that the clear food was sufficient to support normal larval development.

3.2 | Choice test for oviposition on erythritol

Flies showed no significant difference between laying on food substrate with 1 M erythritol vs. control food when given free access to both substrates (Nested ANOVA: $F_{4,18} = 0.93$, $P = .47$).

3.3 | Blue-dyed food test for larval ingestion

This study confirmed the lack of 1M erythritol-contact effects on egg hatching: the number of eggs hatched did not differ significantly between control and 1 M erythritol food treatments on day 1 ($t = 2.07$ $df = 10$, $P = .066$) or on day 2 ($t = 0.64$, $df = 10$, $P = .54$), and the number of larvae visible on the surface did not differ between treatments on day 1 ($t = 1.72$, $df = 10$, $P = .12$) nor on day 2 ($t = 0.86$, $df = 10$, $P = .41$). Larvae fed on the 1M erythritol food: all larvae visible on the food surfaces were moving and burrowing through the food, and all larvae observed had blue-dyed food visible in their

guts through the body wall in both erythritol and sucrose (control) treatments.

3.4 | Adult and larval LC50 studies

The best-fit sigmoidal curve for adult fly LC50 data was:

$$\Pr(\text{surviving at 24 h}) = 89.48/(1+e^{(-([\text{erythritol}]-1.51)/0.17)})$$

This curve was highly significantly fit to the data (r^2 >.99, P = .0004, all model parameters were significant at P < .007). Solving the equation for 50% survival we interpolated an adult fly 24 hr LC50 of 1.56 M erythritol (Figure 2).

The best-fit sigmoidal curve for second-instar larval LC50 data was:

$$\Pr(\text{surviving at 24 h}) = 101.3/(1+e^{(-([\text{erythritol}]-0.60)/0.295)})$$

This curve was highly significantly fit to the data (r^2 > .99, P = .0001, all model parameters were significant at P < .0002). Solving the equation for 50% survival we interpolated a larval fly 24 hr LC50 of 0.59 M erythritol (Figure 2).

3.5 | Reproductive effects of adult feeding on 1M erythritol

Egg laying was depressed for the adult flies that fed on 1 M erythritol. The time course of egg laying suggested egg laying was significantly decreased (relative to controls) on the day flies were fed on 1 M erythritol and for one additional day; egg laying rates rebounded the following day, and were similar control egg laying rates thereafter (Figure 3). Mean total egg production by the females, a measure that is dependent on both female survival and fecundity, varied significantly among treatments ($F_{3,8}$ = 44.9, P < .001). Tukey post hoc tests showed egg output for both day 2 only and day 2-7 flies were significantly reduced compared to controls. Furthermore, eggs laid per female-day, a measure of egg output that is independent of adult survival differences, differed significantly among treatments (Figure 4; $F_{3,8}$ = 48.6, P < .001). Tukey post hoc tests showed egg output for both day 2 only and day 2-7 flies were significantly reduced compared to controls (Figure 4).

Developmental success of the eggs that were laid was not affected by exposure of the parent flies to erythritol. There were no significant differences among treatments in egg hatch rate (GLM $F_{3,8}$ = 1.68, P = .25), pupation rate (no data for [erythritol day 2-7] flies; GLM $F_{2,6}$ = 0.10, P = .90) or adult emergence rate (no data for [erythritol day 2-7] flies; GLM $F_{2,6}$ = 0.87, P = .46).

4 | DISCUSSION

4.1 | Effects of erythritol on egg laying, hatching and larval survival

Female flies readily oviposited on food with erythritol. Flies showed no evidence of avoiding oviposition on food substrate with 1M erythritol, even when given open access to food substrates without erythritol. Egg contact with varying erythritol concentrations did not significantly inhibit egg hatching.

Erythritol (1 M concentration) disrupted development when ingested by *D. melanogaster* larvae; no larvae reared on food with erythritol reached pupation in any study. Larvae feeding on 1M erythritol had significantly reduced lifespans; mean larval longevity from hatching on 1M erythritol was approximately 1.5 days, maximum 3 days (1/38 larvae survived to 3 days) and no larvae on 1 M erythritol developed to pupation. Larval mortality was likely not due to starvation: larvae hatched on the blue-dyed food with 1 M erythritol were seen moving and feeding on the surface in similar numbers to control-fed larvae on days 1 and 2 after egg placement, and all visible larvae had ingested the blue-dyed food medium. Using second-instar larvae on clear food to determine LC50 at 24 hr confirmed that erythritol is lethal to larvae; we estimated an LC50 (24 hr) of approximately 0.6M erythritol for larvae. The comparison with adult fly LC50 suggested larvae were more

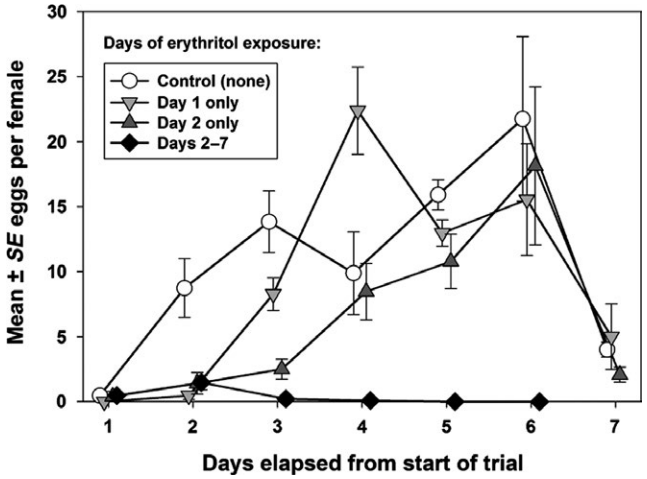

FIGURE 3 Erythritol food treatment effects on the time course of egg production for fruit flies in four feeding treatments, comparing control flies (no erythritol) with flies exposed to food with 1 M erythritol exposure on different days of adult age, as indicated in the graph legend

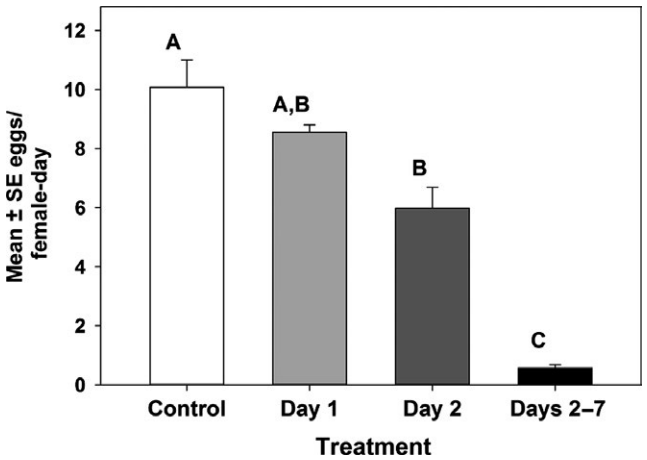

FIGURE 4 Erythritol (1 M) food treatment effects on total egg production by adult fruit flies across seven days of observation. The response variable is per-female egg production. Letters above bars indicate means significantly different according to Tukey post hoc tests (critical alpha=0.05)

sensitive to erythritol ingestion than adults (adult LC50 at 24 hr was approximately 1.6M erythritol). Taken together, these findings suggest erythritol has excellent potential for use in larval-sink methods of insect control (Díaz-Fleischer, Papaj, Prokopy, Norrbom, & Aluja, 2001; Lindh et al., 2015). Because larvae were more sensitive to erythritol, we suggest the effective concentration of erythritol needed in larval-sink traps would be lower than applications for adult control.

4.2 | Erythritol ingestion and adult reproduction

Chemical compounds that suppress reproduction and egg laying can be important components of insect control efforts (Campion, 1972), although some compounds that inhibit insect oogenesis are highly toxic or teratogenic (Kenaga, 1965). Some low-toxicity reproduction inhibitors, such as essential oils, can have undesirable odours or cause human skin irritation (Tripathi, Upadhyay, Bhuiyan, & Bhattacharya, 2009). Erythritol avoids these problems, and our data suggest erythritol has potential for use in control of insect population growth via temporary suppression of reproduction. Egg laying was depressed in adult flies feeding on food with 1 M erythritol. Because male and female flies were housed together, we could not determine if effects on one or both sexes contributed to the reproductive declines, but the timing of egg production suggested egg laying was reduced on the day the adults were exposed to erythritol and for one additional day; afterwards, egg production rebounded to levels similar to control-fed fly egg laying. Erythritol effects on reproduction were rapid, occurring on the first day of exposure and more quickly than increased adult mortality on 1 M erythritol (Baudier et al., 2014). Although exposure of parents to erythritol did not carry over into developmental effects on the offspring they produced, the rapid and persistent effects on reproduction suggest erythritol could affect significant insect control via reduced population growth. Overall treatment-group egg output and egg output per-female were reduced by exposure to erythritol. These patterns suggest brief, temporary exposure to erythritol foods can partially inhibit reproduction, even when the exposure is not sufficient to cause adult mortality.

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AUTHOR CONTRIBUTION

SO'D and DRM designed and supervised the research. KMB and KF collected the data. KMB and SO'D analysed the data. All coauthors contributed to writing the paper.

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